Characterization of chlorophyll *f* synthase heterologously produced in *Synechococcus* sp. PCC 7002

Gaozhong Shen[1], Daniel P. Canniffe[1,†], Ming-Yang Ho[1], Vasily Kurashov[1], Art van der Est[3] John H. Golbeck[1,2], and Donald A. Bryant[1,4] *

[1]Department of Biochemistry and Molecular Biology and [2]Department of Chemistry, The Pennsylvania State University, PA 16802 USA; [3]Department of Chemistry, Brock University, St. Catharines, Ontario, Canada L2S 3A1; and [4]Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717 USA

[†]**Current Address:** Department of Molecular Biology & Biotechnology, University of Sheffield, S10 2TN, UK

***Correspondence:** Dr. Donald A. Bryant, 403C Althouse Laboratory, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802 USA. Phone; 814-865-1992; Fax: 814-863-7024; E-mail: dab14@psu.edu

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Abstract

In diverse terrestrial cyanobacteria Far-Red Light Photoacclimation (FaRLiP) promotes extensive remodeling of the photosynthetic apparatus, including photosystems (PS)I and PSII and the cores of phycobilisomes, and is accompanied by the concomitant biosynthesis of chlorophyll (Chl) *d* and Chl *f*. Chl *f* synthase, encoded by *chlF*, is a highly divergent paralog of *psbA*; heterologous expression of *chlF* from *Chlorogloeopsis fritscii* PCC 9212 led to the light-dependent production of Chl *f* in *Synechococcus* sp. PCC 7002 (Ho et al., *Science* **353**, aaf9178 (2016)). In the studies reported here, expression of the *chlF* gene from *Fischerella thermalis* PCC 7521 in the heterologous system led to enhanced synthesis of Chl *f*. N-terminally $[His]_{10}$-tagged $ChlF^{7521}$ was purified and identified by immunoblotting and tryptic-peptide mass fingerprinting. As predicted from its sequence similarity to PsbA, ChlF bound Chl *a* and pheophytin *a* at a ratio of ~3–4:1, bound β-carotene and zeaxanthin, and was inhibited *in vivo* by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Cross-linking studies and the absence of copurifying proteins indicated that ChlF forms homodimers. Flash photolysis of ChlF produced a Chl *a* triplet that decayed with a lifetime ($1/e$) of ~817 μs and that could be attributed to intersystem crossing by EPR spectroscopy at 90 K. When the $chlF^{7521}$ gene was expressed in a strain in which the *psbD1* and *psbD2* genes had been deleted, significantly more Chl *f* was produced, and Chl *f* levels could be further enhanced by specific growth-light conditions. Chl *f* synthesized in *Synechococcus* sp. PCC 7002 was inserted into trimeric PSI complexes.

Introduction

Because light is their primary energy source for growth, cyanobacteria have evolved complex adaptations and photoacclimation mechanisms that can adjust their photosynthetic apparatus in response to specific light conditions (for reviews, see Chen 2014; Gan and Bryant 2015; Ho et al. 2017c). For example, complementary chromatic acclimation is a well-studied process through which cyanobacteria modify their light-harvesting antenna complexes, specifically the peripheral rods of phycobilisomes, to absorb incident radiation more effectively (Montgomery 2016). The light available to terrestrial cyanobacteria is often strongly filtered by chlorophyll (Chl) *a* because of shading by plants or because of the association of these organisms with soil crusts, dense blooms, and benthic or mat communities. These and other environments can become highly enriched in far-red (FRL; λ = 700 to 800 nm) and near-infrared light. Expanding the wavelength range for oxygenic photosynthesis up to 800 nm would allow cyanobacteria access to about 33% more photons than organisms that are only able to use visible light (400 to 700 nm) (Chen and Blankenship 2011).

Far-Red Light Photoacclimation (FaRLiP) is a recently discovered light acclimation response that occurs when some terrestrial cyanobacteria grow in light wavelengths >700 nm (Gan et al. 2014). Extensive remodeling of their photosynthetic apparatus occurs, and this includes the biosynthesis of new pigments (Chl *f* and Chl *d*) and the assembly of modified photosystem I (PSI), photosystem II (PSII) and phycobilisome (PBS) core complexes (Chen et al. 2012; Gan et al. 2014, 2015; Gan and Bryant 2015; Ho et al. 2017a, b; Li et al. 2016). During FaRLiP, a highly conserved cluster of twenty genes, which encode FRL-specific core subunits of PSII, PSI and PBS, are specifically expressed (Gan et al. 2014; Zhao et al. 2015; Ho et al. 2017b). The FaRLiP gene

cluster also encodes three regulatory proteins, RfpA, RfpB, and RfpC (Gan et al. 2014; Zhao et al. 2015; Ho et al. 2017a,c). RfpA encodes a knot-less red/FRL-dependent phytochrome that acts as a sensor histidine kinase; RfpC is a CheY-like phosphate shuttle, and RfpB is transcriptional activator/response regulator with two CheY input domains (Zhao et al. 2015; Ho et al. 2017a,b,c). Because cyanobacteria that can perform FaRLiP can grow in light environments highly enriched in FRL, these primarily terrestrial cyanobacteria gain a strong selective advantage over organisms that are unable to do so (Gan and Bryant 2015).

Chl *a* is the major photosynthetic pigment in most cyanobacteria, but when FaRLiP strains are grown in FRL, Chl *f* and Chl *d* are also synthesized (Gan et al. 2014, 2015; Airs et al. 2014). These two FRL-absorbing Chls are thought to play important roles in the assembly and function of the PSII and PSI complexes produced in FaRLiP strains growing in FRL. Chls *d* and *f* participate in light harvesting but may also play roles in electron transfer (Gan et al. 2014; Ho et al. 2016, Nürnburg et al. 2018). A mutant that is unable to synthesize Chl *f* is incapable of growth in FRL (Ho et al. 2016). Chl *d* was first discovered more than 75 years ago (Manning and Strain, 1943) and was "rediscovered" in *Acaryochloris marina* in 1996 (Miyashita et al., 1996, 2014; Kashiyama et al. 2008; Chen at al. 2010; Chen 2014; Allakhverdiev et al. 2016). However, the enzyme(s) required to convert the 3-vinyl group of Chl *a* into the 3-formyl group of Chl *d* is unknown (Schliep et al. 2010; Loughlin et al. 2014; Yoneda et al. 2016). Chl *f* was discovered more recently by analyzing the pigments of a cyanobacterium derived from stromatolites (Chen et al. 2010; Chen 2014). Chl *f* synthase was shown to be encoded by the *chlF* gene through reverse genetics and heterologous gene expression. ChlF is a distant paralog of PsbA, the D1 protein of PSII, and thus the *psbA4* gene was renamed *chlF* (Ho et al. 2016). Chl *f* synthesis was shown to be light-dependent, and thus ChlF was proposed to function as a photo-oxidoreductase that oxidizes the 2-

methyl group of Chl *a* (or chlorophyllide (Chlide) *a*) into the 2-formyl group of Chl *f* (Ho et al. 2016). Labeling studies have suggested that the oxygen atom of the 2-formyl group of Chl *f* is derived from dioxygen (Gary et al. 2017).

Establishment of gene expression systems based on multicopy plasmids, the identification of neutral sites in the chromosome, and strong and regulatable promoters (Xu et al. 2011; Pérez et al. 2016) have made *Synechococcus* sp. PCC 7002 (hereafter *Synechococcus* 7002) an ideal model cyanobacterium for functional genomics and other applications in synthetic biology. *Synechococcus* 7002 does not naturally synthesize Chl *f* and is unable to grow in FRL (Gan et al. 2014), so it is a useful platform for studying Chl *f* synthesis and the effects of this long-wavelength-absorbing Chl on photosynthetic complexes. We previously showed that small amounts of Chl *f* can be synthesized when the *chlF* gene from the FaRLiP strain, *Chlorogloeopsis fritschii* PCC 9212 (hereafter *C. fritschii* 9212), is heterologously expressed in *Synechococcus* 7002 (Ho et al. 2016). The *chlF* gene is found in all cyanobacterial strains capable of FaRLiP and to date is always located in the FaRLiP gene cluster (Ho et al. 2016). When cyanobacterial strains capable of FaRLiP are grown in FRL, differences have been noted in their cellular contents of Chl *d* and Chl *f*; furthermore, associated differences in the absorption and fluorescence emission spectra of the corresponding cells were also noted (Gan et al. 2014, 2015; Ho et al. 2017a,b,c). These observations suggested that the cellular contents of Chls *d* and *f* might vary, and that the amounts and/or activities of ChlF might differ substantially among FaRLiP strains (Gan et al. 2015, Zhao et al. 2015; Ho et al. 2017b). Thus, we concluded that it could be interesting to test whether another Chl *f* synthase might exhibit better activity when heterologously produced in *Synechococcus* 7002.

Chl *f* synthase (ChlF) belongs to a highly divergent, "super-rogue" clade of the PsbA superfamily (Murray 2012; Cardona et al. 2015; Ho et al. 2016). As shown in sequence alignment

comparisons, ChlF is distinguished from other PsbA-like (D1) subunits of PSII reaction centers both in the N-terminal and especially the C-terminal regions of the protein. ChlF lacks residues essential for binding the water-oxidizing $Mn_4Ca_1O_5$ cluster (Ho et al. 2016). However, ChlF is predicted to bind Chl *a* and pheophytin (Pheo) *a* like PsbA (Murray 2012; Cardona et al. 2015). Purification and characterization of ChlF could verify these predictions and provide new insights into the structure and function of ChlF in the light-driven Chl *f* synthesis reaction. In PSII reaction centers, D1 (PsbA) and D2 (PsbD) form the heterodimeric core and bind the essential cofactors for electron transport in PSII (Ferreira et al. 2004; Umena et al. 2011; Shen 2015; Barber 2017). Heterologous expression of *chlF* in a null mutant lacking PsbD1 and PsbD2 should confirm whether ChlF requires PsbD or PSII for activity.

In this study we report the purification and initial characterization of Chl *f* synthase, which was obtained through heterologous expression of *chlF* gene from two different cyanobacterial strains capable of FaRLiP, *C. fritschii* 9212 and *Fischerella thermalis* PCC 7521 (hereafter *F. thermalis* 7521). Expression of $[His]_{10}$-tagged *chlF* allowed Chl *f* synthase to be purified by immobilized metal-chelate affinity chromatography and studied biochemically. The substantially enhanced synthesis of Chl *f* in a strain lacking PsbD (D2) demonstrates that the Chl *f* synthase activity of $ChlF^{7521}$ is not dependent on any interaction with PsbD or on PSII activity. The synthesis of Chl *f* could also be enhanced by specific growth light conditions for *Synechococcus* 7002, and interestingly, we show that some Chl *f* was incorporated into PSI complexes of this strain that cannot perform FaRLiP.

Materials and Methods

Strains and growth conditions

The wild-type strain of *Synechococcus* sp. PCC 7002 (Rippka et al. 1979) and strains for modified heterologous expression of the *chlF* gene were grown in liquid A^+ medium under standard conditions at 38°C; cultures were sparged with 1% (v/v) CO_2 in air as previously described (Ludwig and Bryant 2011). *Synechococcus* 7002 strains in which the *psbD1* and *psbD2* genes have been deleted are light sensitive and were grown under low-irradiance conditions (~10 µmol photons m^{-2} s^{-1}). The A^+ medium for these mutants lacking PSII activity was supplemented with 20 mM glycerol, which served as the primary carbon and electron source for growth (Lambert and Stevens 1986). For all genetically modified strains, antibiotics were added as required at the following concentrations: gentamycin (50 µg ml^{-1}); spectinomycin (100 µg ml^{-1}); and kanamycin (100 µg ml^{-1}). Cultures of *F. thermalis* 7521 and *C. fritschii* 9212 were grown in B-HEPES medium (Dubbs et al. 1991), a modified BG11 medium which is buffered at pH 8 with 4.6 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), as described previously (Gan et al. 2015).

Cool white fluorescent lights provided an irradiance of 250 µmol photons m^{-2} s^{-1} for standard white-light (WL) or ~10 µmol photons m^{-2} s^{-1} for low-light (LL) growth conditions. Different light intensities were achieved either by varying the number of fluorescent tubes or by using neutral density filters. Green light (GL; 11 µmol photons m^{-1} s^{-1}) and red light (RL; 18 µmol photons m^{-1} s^{-1}) were provided with green (GamColor_660) and red (GamColor_250) light-transmitting filters from Parlights, Inc (Frederick, MA, USA), as described (Gan et al., 2014, 2015). For growth of liquid cultures under far-red light (FRL) conditions, FRL (25-30 µmol photos m^{-2} s^{-1}) was provided with 720-nm LED light panels (L720-06AU) (Marubeni, Santa Clara, CA,

USA) or a combination of green- and red-light-transmitting filters, as described previously (Gan et al. 2014, 2015; Ho et al. 2017a).

Construction of *Synechococcus* 7002 strains for heterologous expression of *chlF* genes

DNA fragments encoding the *chlF* genes from *C. fritschii* 9212 and *F. thermalis* 7521 were amplified by polymerase chain reaction (PCR) using Phusion DNA polymerase (New England Biolabs, Ipswich, MA). The resulting amplicons were digested with NdeI and BamHI, and the products were cloned into the pAQ1Ex-P_{cpcBA} (**Table 1**) shuttle vector as previously described (Xu et al. 2011; Ho et al. 2016). This resulted in the addition of the coding sequence for the production of an N-terminal $[His]_{10}$-tag to ChlF. After verification by DNA sequencing, the resulting plasmid vectors, pAQ1Ex*::chlF*^{9212} and pAQ1Ex*::chlF*^{7521} (**Table 1**) were transformed into cells of the *Synechococcus* 7002 to generate strains expressing the *chlF* gene from *C. fritschii* 9212 or *F. thermalis* 7521, respectively. *Synechococcus* 7002 was transformed as previously described (Frigaard et al. 2004).

To construct a *Synechococcus* 7002 strain lacking *psbD1* and *psbD2*, two plasmids were constructed from pUC19 to allow independent inactivation of the *psbD1* and *psbD2* genes. The *ΔpsbD1*::*aadA* construct was made by amplifying and cloning the flanking sequences of the *psbD1* gene with a DNA fragment encoding *aadA,* which confers resistance to spectinomycin, replacing the *psbD1* coding sequence (**Table 1**) (Frigaard et al. 2004). Transformation of *Synechococcus* 7002 with the *ΔpsbD1*::*aadA* plasmid resulted in a *Synechococcus* 7002 *ΔpsbD1::aadA* mutant. The *ΔpsbD2*::*aphAII* construct was generated by amplifying the flanking sequences of the *psbD2* gene and replacing the *psbD2* coding sequences with a DNA fragment encoding *aphAII,* which confers resistance to kanamycin (Frigaard et al. 2004). Transformation of *Synechococcus* 7002

ΔpsbD1::aadA mutant cells with the *ΔpsbD2*::*aphAII* plasmid resulted in a spectinomycin- and kanamycin-resistant *Synechococcus* 7002 strain lacking *psbD1* and *psbD2* and devoid of PSII activity (**Table 1**). Segregation of the *ΔpsbD1 ΔpsbD2* transformants was achieved by restreaking colonies on A^+ agar plates supplemented with 20 mM glycerol and additions of 100 µg spectinomycin ml^{-1} and 100 µg kanamycin ml^{-1}. Full segregation of the *Synechococcus* 7002 mutants lacking *psbD1* and/or *psbD2* was verified by PCR analysis, and the absence of functional PSII was further verified by the fact that the resultant mutant could not grow photoautotrophically, was sensitive to high light, and had an altered fluorescence emission spectrum at 77K (see Results). To obtain a strain devoid of PSII activity and PsbD that expressed the *chlF*^{7521} gene, *Synechococcus* 7002 *ΔpsbD1ΔpsbD2* mutant cells were transformed with the pAQ1Ex*::chlF*^{7521} expression vector (**Table 1**).

Generation of ChlF variants by site-directed substitution mutagenesis

To construct variant ChlF^{9212} proteins with site-specifically mutated residues, the *chl*F^{9212} gene was first cloned into plasmid pUC19. A pair of partially complementary mutagenic primers were designed to change the codon for the specific amino acid residue substitution. PCR amplification was carried out using Q5 Hot-Start High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). After PCR, the amplified DNA was added directly to a unique Kinase-Ligase-DpnI (KLD) enzyme mix (New England Biolabs, Ipswich, MA) for rapid circularization and removal of the unmodified plasmid template. Following transformation into *E. coli* and colony screening, plasmid DNA was isolated for DNA sequencing to verify that the *chlF*^{9212} gene was only mutated as intended. The mutated *chlF*^{9212} gene was subcloned into the pAQ1Ex-P_{cpcBA} shuttle vector,

which was transformed into *Synechococcus* 7002 for heterologous expression as described previously (**Table 1**; Xu et al. 2011; Ho et al. 2016)

Purification of heterologously expressed ChlF by immobilized metal-chelate affinity chromatography

Heterologously produced ChlF^{7521} was purified from WT and *ΔpsbD1ΔpsbD2* mutant cells of *Synechococcus* 7002 that harbored plasmid pAQ1Ex*::chlF*^{7521} (**Table 1**). Cells were harvested by centrifugation and were resuspended in cell resuspension buffer (50 mM HEPES, pH 7.4, 10 mM $CaCl_2$, and 10 mM $MgCl_2$). Cells were lysed by three passages through a chilled French pressure cell operated at 138 MPa. After centrifugation at 6,900 × *g* to remove unbroken cells and large cell debris, total membranes were pelleted by ultracentrifugation (126,000 × *g*) and resuspended in membrane buffer A (50 mM HEPES, pH 7.4, 300 mM NaCl, 20 mM imidazole). Membranes were diluted to 0.4 mg Chl ml^{-1} in membrane buffer and were solubilized by addition of *n*-dodecyl-β-D-maltoside (DM) to a final concentration of 1% (w/v) at 4 °C. Solubilized membranes were separated from insoluble debris by centrifugation (24,000 × *g* for 20 min). Prior to immobilized metal-chelate affinity chromatography (IMAC), solubilized membranes were diluted with five volumes of the membrane buffer A, and the resulting solution was loaded onto a column that was packed with high density Ni-nitrilotriacetic acid (NTA) agarose resin (Gold Biotechnology, St. Louis, MO) and equilibrated with the binding buffer B (50 mM HEPES, pH 7.4, 300 mM NaCl, 20 mM imidazole, 0.1% (w/v) DM). The column was washed with two column volumes of binding buffer and three column volumes with wash buffer (50 mM HEPES, pH 7.4, 300 mM NaCl, 50 mM imidazole, 0.1% (w/v) DM). The $[His]_{10}$-ChlF protein was subsequently eluted with elution buffer (50 mM HEPES, pH 7.4, 300 mM NaCl, 250 mM imidazole, 0.1% (w/v) DM). The solution

containing ChlF was dialyzed against sample buffer (50 mM HEPES, pH 7.4, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.05% (w/v) DM, 5% (w/v) glycerol), concentrated by using Millipore Centriprep 30K Centrifugal Filter Devices (EMD Millipore, Darmstadt, Germany), and stored at –80 °C until required.

Cross-linking, gel electrophoresis and immunoblotting

The method for protein crosslinking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) has been described previously (Li et al. 2006). Methods for polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) and immunoblotting have been described (Shen et al. 2002). For immunoblotting, proteins were transferred electrophoretically onto 0.2-µm nitrocellulose membranes using a semi-dry transfer cell (BioRad, Hercules, CA, USA). Immunodetection of the $[His]_{10}$-ChlF was achieved using rabbit antibodies to a $[His]_6$ epitope, which were conjugated to horseradish peroxidase (Rockland, Limerick, PA, USA). Immunolabelled proteins were detected using Pierce® enhanced chemiluminescence reagents (Thermo Fisher Scientific, Waltham, MA, USA).

In-solution trypsin digestion and LC-MS-MS protein identification

In-solution digestion of proteins with trypsin and subsequent LC-MS-MS analyses were performed at the PARC Mass Spectrometer Facility at Washington University in St. Louis. The raw data from the LC-MS-MS analysis was directly loaded into PEAKS (v 7.0, Bioinformatics Solution Inc., Waterloo, ON, Canada) to perform database searches against the total proteome of *Synechococcus* 7002, to which the ChlF sequence from *F. thermalis* 7521 had specifically been added.

Isolation of trimeric PSI complexes

PSI complexes were purified from *Synechococcus* 7002 wild type (WT) and from the WT and *ΔpsbD1ΔpsbD2* mutant cells harboring plasmid pAQ1Ex*::chlF*^{7521} by following procedures described previously (Shen et al. 2002, 2016). Cells were harvested and resuspended in MES buffer (50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5, 10 mM $CaCl_2$ and 10 mM $MgCl_2$). Membranes from these strains were isolated and solubilized as described above. Solubilized membranes were loaded onto 5 to 20% (w/v) sucrose gradients containing 0.1% DM and were centrifuged for about 18 h at 108,000 × *g*. Green-colored fractions containing trimeric PSI complexes were collected from the lowest regions of the sucrose gradients, dialyzed against MES buffer and concentrated using the Millipore Centriprep 100K Centrifugal Filter Devices (EMD Millipore, Darmstadt, Germany). The concentrated PSI complexes were purified further by ultracentrifugation on similar sucrose gradients lacking added DM. Purified PSI complexes were resuspended in MES buffer containing 0.05% (w/v) DM and 5% (w/v) glycerol.

Pigment extraction and HPLC analysis

Pigments were extracted from cells, purified ChlF protein preparations and PSI complexes by sonication in the dark with acetone/methanol (7:2, v/v). To extract pigments from cells of *Synehcococcus* 7002 strains, cells were harvested from liquid cultures by centrifugation, washed once in 50 mM HEPES buffer, pH 7.2, and resuspended in the same buffer. Cells equivalent to 1.0 ml with an OD_{750} = 2.0 were pelleted and extracted with sonication in acetone/methanol (7:2 (v/v), 250 µl). The pigment extracts were filtered using Whatman™ 0.2-µm polytetrafluoroethylene syringe filters (GE Healthcare Life Sciences, Boston, MA, USA) and an aliquot (100 µl) of the extract solution was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) on an Agilent 1100 HPLC system with a Model G1315B diode-array detector (Agilent

Technologies, Santa Clara, CA) equipped with an analytical Discovery C18 column (4.6 mm × 25 cm) (Supelco, Sigma-Aldrich, St. Louis, MO), following the solvent methods described previously (Gan et al. 2014, 2015). An alternative HPLC analysis method was sometimes used and has been described previously (Ortega-Ramos et al. 2018). Pigment extracts were filtered and buffered by addition of 0.1 volume of 1.0 M ammonium acetate before injection onto the HPLC column. Solvents A and B were 64:16:20 (v/v/v) methanol/acetone/H_2O and 80:20 (v/v) methanol/acetone, respectively. To detect species of Chl *a*, *d*, and *f* as well as carotenoids, the absorbance spectra of all eluted compounds were collected between 350 and 900 nm at 0.5-s intervals. The HPLC data were processed using Agilent ChemStation software (revision B.02.01-SR1 6100 series).

Absorption and fluorescence spectroscopy and pigment content determination

Absorption spectra were measured using a Cary 14 spectrophotometer that was modernized for computerized operation, data collection and analysis by On-Line Instrument Systems, Inc. (Bogart, GA, USA). Fluorescence emission spectra were measured at 77K using an SLM Model 8000C spectrofluorometer modernized for computerized, solid-state operation by On-line Instrument Systems Inc., (Bogart, GA USA) as described (Shen et al. 2008). For measuring the fluorescence emission from Chl-protein complexes, the excitation wavelength was 440 nm, which selectively excites Chls.

Pigments were quantified by absorption spectroscopy. Equations based on published molecular extinction coefficients (Li et al. 2012) for calculating Chl *a* and Chl *f* concentrations can be found in Li et al. (2014). Chl *a* and Chl *f* concentrations were also sometimes determined from absorption spectra of extracts from the Q_y absorption band of Chl *a* at 665 nm and of Chl *f* at 707 nm by using the molar extinction coefficients in methanol for Chl *a* (70.54 mM^{-1} cm^{-1}; Lichtenthaler 1987) and Chl *f* (71.11 mM^{-1} cm^{-1}; Li et al. 2012). The Chl *a*, Pheo *a*, and carotenoid

contents of ChlF preparations were calculated as described previously (Eijckelhoff and Dekker 1997). The concentrations of β-carotene (and zeaxanthin) were determined from absorbance spectra using the extinction coefficient 120.84 mM^{-1} cm^{-1} at 480 nm. The concentration of pheophytin *a* was determined from absorbance spectra using its extinction coefficient at 412 nm of 112.57 mM^{-1} cm^{-1}.

Flash-induced transient absorption spectroscopy

Transient absorbance changes at 830 nm were measured at room temperature with a laboratory-built, dual-beam spectrometer described earlier (Vassiliev et al. 1997; Hays et al. 1998) with the following modifications. A 100 mW, 830-nm laser diode (Crystalaser, model number DL830-100-O, Reno, NV) was split into a measuring and reference beam using a 70:30 beam splitter and collimators (F240FC-780, Thor Labs, Newton, NJ). After passing through the sample, the beams were directed to a balanced amplified photodetector (PDB460A, Thor Labs, Newton, NJ) with lenses and fiber optic cables (M74L01, Thor Labs, Newton, NJ). Each collimating lens had an 830-nm interference filter (FL830-10, Thor Labs, Newton, NJ) in front of it, and the sample arm collimator had an additional 532-nm notch filter (NF533-17, Thor Labs, Newton, NJ) to remove stray actinic light from the pump laser. The amplified differential signal was processed with a 1-GHz bandwidth, 8-bit, 2 GS/s, PCI card analog-to-digital converter (NI-5154, National Instruments, Austin, TX). A 532-nm, frequency-doubled Nd:YAG laser (Minilite II, Continuum, San Jose, CA) with a 7-ns nominal pulse-width was used as the actinic light source. The flash power was 34 mJ, and the frequency of the flashes was 0.1 Hz (1 flash per 10 sec). The light intensity study was carried out using assorted optical filters with 3.2%, 11.2%, 34.6%, 52% and 70% transmittance. The 78-mW point was measured using a Quanta-Ray DCR-11 (Spectra-Physics, Santa-Clara, CA) laser at its highest power.

Purified ChlF protein was diluted to 20 µg Chl ml^{-1} in 50 mM HEPES, pH 7.4, containing 10 mM sodium ascorbate, 4 µM 2,6-dichlorophenolindophenol (DCPIP) and 0.02% (w/v) DM. The data were plotted in Igor Pro (Wavemetrics, Lake Oswego, OR) and decomposed using the CONTIN algorithm as described for PSI in (Kurashov et al., 2018). The data were refitted on the experimental plot using a multi-exponential fit algorithm with kinetic inputs from CONTIN.

Transient X-band EPR spectroscopy

Transient EPR measurements were performed using a modified Bruker E300 EPR spectrometer at X-band and 90 K as described in detail in (Ferlez et al., 2016). The spectrum was extracted from the time/field/amplitude data set by calculating the average EPR signal at each field point in a 1 µs time window starting 1 µs following the laser flash. The data were plotted and analyzed using Igor Pro (Wavemetrics, Lake Oswego, OR).

Results

Chl *f* production in *Synechococcus* 7002

The cyanobacterium *Synechococcus* 7002 does not naturally synthesize Chl *f*, so it is an excellent platform to test the heterologous expression of *chlF* genes. A previous study showed that heterologous expression of the *chlF* gene from *C. fritschii* 9212 led to a low level of Chl *f* synthesis in *Synechococcus* 7002 cells (Ho et al. 2016; also see **Fig. 1**). To verify the function of ChlF, and to determine whether expression of an alternative *chlF* gene might lead to enhanced Chl *f* synthesis in *Synechococcus* 7002, the *chlF* gene from the thermophilic cyanobacterium *F. thermalis* 7521 was expressed from the strong P_{cpcBA} promoter from *Synechocystis* sp. PCC 6803 (Xu et al. 2011; Zhou et al. 2014). As shown in **Fig. 1**, a strain expressing the $chlF^{7521}$ gene accumulated about 10-fold more Chl *f* than a strain expressing the $chlF^{9212}$ gene. This result establishes that the *chlF* gene is an essential component of Chl *f* synthase and also suggests that the $ChlF^{7521}$ protein level and/or enzyme activity might be considerably higher than those in a strain producing $ChlF^{9212}$. Based upon the assumption that the increased Chl *f* levels in *Synechococcus* 7002 would be positively correlated with the amounts (and activity) of ChlF in cells, growth conditions, primarily light intensity and color were varied to identify optimal conditions for Chl *f* synthesis in *Synechococcus* 7002. Further testing showed that the highest Chl *f* levels accumulated in cells that were grown at low light intensities (<50 µmol photons m^{-1} s^{-1}; data not shown).

Affinity purification of $ChlF^{7521}$

Total membranes isolated from the WT strain of *Synechococcus* 7002 harboring plasmid pAQ1-Ex-P_{cpcBA}::$chlF^{7521}$ were solubilized with 1% (w/v) DM, and the resulting solution was fractionated by IMAC. Different concentrations of imidazole and washing volumes were tested to

achieve the highest purity of $[His]_{10}$-$ChlF^{7521}$. As shown in **Fig. 2a,** purified $[His]_{10}$-$ChlF^{7521}$ had an apparent mass of ~44 kDa upon SDS-PAGE, which is consistent with its predicted mass of 44.2 kDa. Immunoblotting with rabbit antibodies raised against a $[His]_6$-tag epitope confirmed that the purified protein also carried a poly-[His]-tag as expected (**Fig. 2b**).

To confirm the identity of purified ChlF and to investigate whether any specific protein(s) copurified with $[His]_{10}$-ChlF, two independently purified preparations of ChlF were subjected to in-solution digestion with trypsin and subsequent LC-MS-MS analysis of the resulting tryptic peptides. As shown in **Fig. S1** a total of six $ChlF^{7521}$ peptides were identified by LC-MS-MS analysis for the two preparations, and two of those peptides were identified in both analyses. It should be noted that two peptides, including the C-terminal peptide, were derived from the C-terminal region of $ChlF^{7521}$. These analyses show that, unlike the PsbA (D1) protein of PSII (Nixon et al. 1992), $ChlF^{7521}$ probably does not require activation by proteolytic processing at the C-terminus. Several other contaminating *Synechococcus* 7002 proteins that co-purified with $ChlF^{7521}$ were identified, but they mostly had very low scores because only 1 to 3 peptides were identified (data not shown). For example, the highly abundant, Chl-binding PsaA and PsaB subunits of PSI were detected as contaminants, but in spite of their very large sizes, only one or three unique peptides were identified these proteins. PsbB (CP47) of PSII was the only other Chl-binding protein detected, although only a single peptide was identified in spite of the large size of this protein (507 aa; 55.8 kDa). Notably, the core subunits of PSII, including PsbA (D1) and PsbD (D2), were not detected in highly purified preparations of $ChlF^{7521}$. Correspondingly, ChlF was never detected by tryptic-peptide mass fingerprinting of highly purified PSII complexes from of *Synechococcus* 7335 and *C. fritschii* 9212 (M.-Y. Ho and D. A. Bryant, in preparation). The results show that $ChlF^{7521}$ does not form stable complexes with other proteins of the *Synechococcus* 7002

photosynthetic apparatus and that it is not a subunit of FRL-PSII in *Synechococcus* 7335 and *C. fritschii* 9212.

Pigment content of ChlF^{7521}

Purified ChlF^{7521} was pale yellow-green in color (**Fig. 3a**), which suggested that this protein might bind Chl and carotenoids. The absorbance spectrum of the protein, with maxima at 437 nm and 674 nm and a shoulder at 490 nm, was consistent with that of a Chl *a*-binding protein. The fluorescence emission spectrum at 77K (**Fig. 3b**) was asymmetric with a peak centered at ~680 nm. The fluorescence emission between 700-750 nm after excitation of Chl at 440 nm suggested that some minor amounts of far-red-absorbing Chl molecules might be present in purified ChlF^{7521} (see below).

Pigments were extracted from preparations of ChlF^{7521} and subjected to analysis by reversed-phase HPLC. As shown for a typical preparation in **Fig. 4**, ChlF^{7521} contains both Chl *a* and Pheo *a*; the latter was identified by its elution time as well as its characteristic absorption spectrum (**Fig. 4**, inset). As shown in **Fig. S2**, two carotenoids were detected in purified ChlF^{7521}. Based upon their elution times and absorbance properties, they were identified as β-carotene and zeaxanthin (Graham and Bryant 2009). The ratio of Chl *a*: β-carotene: zeaxanthin was approximately 8.3:0.5:0.4. A very small amount of Chl *f* was also detected in the pigments extracted from purified ChlF^{7521} (data not shown). Based on estimates from the peak areas for Chl *f* and Chl *a* in the HPLC profiles at 680 nm, ChlF^{7521} is estimated to bind 25 to 29 Chl *a* molecules per Chl *f*. This indicates that Chl *f* is probably not a stable component of the enzyme. The Pheo *a* content was estimated from the peak area at 408 nm in the HPLC elution profile. The estimated

ratio of Chl *a*: Pheo *a* was ~3 to 4 : 1, which was determined by averaging the results from several independent preparations of ChlF^{7521}.

Cross-linking of ChlF^{7521}

To determine whether the purified ChlF^{7521} forms oligomers, purified [His]$_{10}$-ChlF was treated with the zero-length cross-linking reagent, EDC, and the cross-linked products were analyzed by SDS-PAGE and immunoblotting. As shown in **Fig. S3a**, prior to treatment with EDC, only a single polypeptide with a mass of about 44 kDa was detected for [His]$_{10}$-ChlF by immunoblotting and Coomassie blue staining . This 44-kDa polypeptide was still detected after EDC treatment, but an additional species with an estimated mass of about 90 kDa was also observed both by Coomassie-blue staining as well by immunoblotting (**Fig. S3a, b**). Based on this result, as-isolated ChlF^{7521} apparently exists as homodimers in solution.

Enhanced Chl *f* production in mutant cells lacking PsbD (D2) and PSII

PsbA forms a heterodimer with PsbD that binds the essential electron transfer cofactors in PSII complexes (Shen 2017). To determine whether the PsbD (D2 protein) of PSII is required for the Chl *f* synthase activity of ChlF, a *Synechococcus* 7002 strain lacking PsbD was constructed. Like most other cyanobacteria, *Synechococcus* 7002 has two *psbD* genes, *psbD1* and *psbD2,* that encode identical polypeptides (Gingrich et al. 1990). Deletion of the *psbD1* and *psbD2* genes resulted in a light-sensitive, *ΔpsbD1 ΔpsbD2* mutant strain that was no longer able to grow photoautotrophically and that required the addition of glycerol to the growth medium (data not shown). As shown by the low-temperature fluorescence emission spectrum of this mutant at 77K, (**Fig. S4**), this strain could not correctly assemble and accumulate PSII core complexes. This is

reflected by the reduced fluorescence emission at 684 nm and the complete loss of the fluorescence emission band at 694 nm, both of which are characteristic of PSII in *Synechococcus* 7002 (Shen and Bryant 1995; Zhang et al. 2014). Note that the residual fluorescence emission at ~683 nm results from fluorescence emission from the terminal emitters, ApcD and ApcE, of phycobilisomes (Bryant 1991; Sidler 1994; Shen and Bryant 1995).

To determine whether Chl *f* synthesis can occur in a strain that lacks PsbD and active PSII, the pAQ1Ex*::*P_{cpcBA}*::chlF*^{7521} expression vector was transformed into the *ΔpsbD1 ΔpsbD2* mutant strain of *Synechococcus* 7002. Pigments were extracted and analyzed by HPLC from cells of the resulting strain that had been grown under low intensity white light. As shown in **Fig. 5**, the absence of PsbD and PSII activity did not inhibit Chl *f* synthase activity; in fact, the mutant strain produced ~2-fold more Chl *f* than the equivalent WT strain expressing the *chlF*^{7521} gene (compare **Figs. 1**, **5 and S5)**.

Previous studies had suggested that Chl *f* levels were lowest in cells grown at relatively high irradiance in white light (Ho et al. 2016). Thus, we tested whether different light colors and intensities might affect the synthesis and accumulation of Chl *f* (**Fig. S5**). Cells grown under low intensity white light had the lowest Chl *f* levels. Interestingly, a series of three minor peaks, which also had the absorption spectrum of Chl *f,* were noted at elution times shorter than that of Chl *f* esterified with phytol. We speculate that these peaks represent Chl *f* esterified with geranylgeraniol, dihydrogeranylgeraniol, tetrahydrogeranylgeraniol, respectively. Cells grown in red or green light had Chl *f* contents that were 2.1 to 2.4-fold higher than cells grown in low intensity white light. The highest Chl *f* content, which was ~3.1-fold higher than cells grown in low intensity white light, occurred in cells grown in FRL. Under optimal conditions, the Chl *f*

content of the *Synechococcus* 7002 cells was between 3 and 4% of the total Chl content. This corresponds to about 50% of the Chl *f* contents of FaRLiP strains grown in FRL.

PsbA and PsbD of PSII each have binding sites for one plastoquinone molecule, and PSII activity is inhibited when 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is bound to the Q_B plastoquinone binding site on PsbA (Mattoo et al. 1981). To determine whether ChlF might use plastoquinone as an electron acceptor, the Chl *f* synthesis was compared in cells of the PsbD-less mutant strain of *Synechococcus* 7002 in the presence and absence of the plastoquinone analog, DCMU (DCMU does not inhibit the growth of these cells which do not have PSII activity). As shown in **Fig. 5**, the level of Chl *f* was much lower (~80% less) in the presence of 10 µM DCMU. This result suggests the Chl *f* synthase activity of ChlF^{7521} is sensitive to DCMU, which is consistent with the hypothesis that PQ acts as an electron acceptor during Chl *f* synthesis (Ho et al. 2016).

Site-directed mutagenesis of the *chlF* gene

As previously shown in amino-acid sequence comparisons of PsbA and ChlF (Murray 2012; Cardonna et al. 2015; Ho et al. 2016), ChlF has a conserved Tyr residue at position 183 that is equivalent to the redox-active Tyr_Z (Tyr171) of PsbA of PSII (Ho et al. 2016). To determine whether Tyr183 might play an important role in ChlF activity, this residue was replaced by Phe to produce a Y183F variant of ChlF^{9212}. Unexpectedly, as shown in **Fig. S6**, the *Synechococcus* 7002 strain producing the Y183F variant of ChlF^{9212} actually synthesizes about 2-fold more Chl *f* than the WT variant. This result indicates that, although Tyr183 is conserved in ChlF, it may not play an essential role in the photochemical activity of ChlF in Chl *f* synthesis.

Flash-induced absorbance changes in the near-IR

To investigate whether light-induced photochemistry could be detected in purified ChlF^{7521}, flash-induced absorbance changes were recorded at 830 nm. **Fig. 6 (inset, left)** shows the experimental data for purified ChlF in the presence of 1 mM sodium ascorbate. The laser flash causes a rapid (faster than the resolution of our instrument) increase in absorbance followed by a slower multiexponential decay. A decomposition by the CONTIN algorithm (**Fig. 6, main body)** revealed five kinetic phases. The major kinetic phase has a lifetime of 817 μs, and its contribution is 77% of total amplitude of the signal at an excitation intensity of 34 mW. A flash intensity *vs.* amplitude study (**Fig. 6, inset, right**) of this kinetic phase showed a linear increase up to the maximum power of the excitation laser. This behavior is characteristic of the decay of a chlorophyll excited triplet state rather than charge recombination, which should saturate at the laser powers available. The four minor components (**Fig. 6, main body**) have lifetimes (and amplitudes) of 28 μs (6%), 11 ms (6%), 161 ms (7%) and 2.1 s (4%) at an excitation intensity of 34 mW. The origins of the four minor kinetic phases are unknown and are currently under further study.

Transient X-band EPR spectroscopy of ChlF^{7512}

Because the behavior of the major kinetic phase detected optically suggests that it is due to chlorophyll excited triplet state, we measured the transient EPR spectrum of ChlF. The EPR spectra of triplet states are easily distinguished from radical pairs and the polarization pattern(s) generated by intersystem crossing (ISC) is(are) markedly different from that resulting from radical pair recombination (Thurnauer 1979). **Fig. 7** shows the transient EPR spectrum of ChlF at 90 K **(black trace)**. The ~600 mT width of the spectrum and overall polarization pattern E/E/E/A/A/A (A = absorption, E = emission) indicates that the majority of the signal is derived from a triplet

state populated by intersystem crossing. The red trace is a simulation of the experimental spectrum using the zero-field splitting parameters D (2.71 x 10^{-2} cm^{-1}) and E (3.3 x 10^{-3} cm^{-1}) which are typical of Chl *a* (Thurnauer 1979). The simulated spectrum has been calculated as the sum of net and multiplet polarization from intersystem crossing (green and blue traces, respectively) and polarization generated from radical pair recombination (pink trace). As can be seen, the polarization pattern is dominated by the multiplet polarization generated by ISC with a weak net contribution as expected at high magnetic field. The small contribution from the polarization generated by radical pair recombination (**Fig. 7, pink trace)** might be due a small amount of PSI in the sample**.** However, the quality of the fit is not sufficient to accurately determine the size of this contribution. There are also weak features at the extremes of the experimental spectrum that are not fitted in the simulation and that are derived from a triplet species with a larger D-value, possibly Chl *f* or a carotenoid. Overall, the data indicate that the experimental spectrum is predomimantly from the triplet state of Chl *a* populated by ISC, which is consistent with the time-resolved optical study (see above).

Insertion of Chl *f* molecules in PSI trimers of *Synechococcus* 7002

Having improved the conditions for synthesis of Chl *f* dramatically in *Synechococcus* 7002, it was of interest to determine whether any of the Chl *f* was associated with PSI, the major Chl-binding complex in cyanobacterial cells (Fujita and Murakami 1987). **Fig. 8a** shows a comparison of the absorption spectra for trimeric PSI complexes isolated from *Synechococcus* 7002, the PsbD-less mutant of *Synechococcus* 7002 expressing $chlF^{7521}$ and grown in FRL as described above, and trimeric PSI complexes isolated from *F. thermalis* 7521 cells grown in FRL. The absorption spectra of native PSI trimers isolated from cells heterologously expressing $chlF^{7521}$ in the absence

of PsbD clearly show enhanced absorption beyond 700 nm, although the amplitude of that absorption is lower and does not extend as far towards 800 nm as observed PSI complexes isolated from *F. thermalis* 7521 cells grown in FRL. The spectra of the acetone/methanol extracts as well as reversed-phase HPLC analysis of the three trimeric PSI complexes confirmed that these PSI complexes contained about 3% Chl *f* compared to the 8% found in FRL trimeric PSI from *F. thermalis* 7521 cells grown in FRL. *Synechococcus* 7002 PSI complexes normally have a fluorescence emission maximum at 714 nm at 77K (**Fig. 8b**), but the 77K emission maximum of complexes from cells expressing the *chlF*^{7521} gene occurred at 718 nm. Although this spectrum is detectably red-shifted, it is not as red-shifted as the emission spectrum of *F. thermalis* 7521 PSI complexes isolated from cells grown in FRL (**Fig. 8b**). These results show that the Chl *f* content of trimeric PSI complexes was similar to that of whole cells, but the spectroscopic properties of the complexes containing this amount of Chl *f* were quite different from complexes produced by a FaRLiP strain grown in FRL.

Discussion

In cyanobacteria that perform FaRLiP, Chls *f* and *d* are essential components for the remodeling of PSI and PSII that is known to occur when cells are grown in FRL (Gan et al. 2014 15; Airs et al. 2014; Ho et al. 2016). Null mutants of *chlF* in *C. fritschii* 9212 and *Synechococcus* sp. PCC 7335 lack Chl *f* synthase, are unable to synthesize Chl *f,* are unable to assemble FRL-PSI and FRL-PSII, and thus, these mutants are severely impaired in their capacity to grow in FRL (Ho et al. 2016). As reported previously and further confirmed in this study (**Fig. 1**), *Synechococcus* 7002 can synthesize Chl *f* when the *chlF* genes from two FaRLiP strains, *C. fritschii* 9212 and *F. thermalis* 7521, are heterologously expressed. By expressing the *chlF* gene from *F. thermalis* 7521, deleting *psbD1* and *psbD2* to inactivate PSII, and modifying the light conditions for growth for the resulting strain, we were able to increase the amount of Chl *f* synthesized in *Synechococcus* 7002 cells by as much as ~70-fold by comparison to the original construct expressing $chlF^{9212}$ (Ho et al. 2016). The results presented here show that ChlF is uniquely required for the synthesis of Chl *f* in *Synechococcus* 7002 cells.

Similar to the oxidation of two water molecules to produce dioxygen by PSII, the oxidation of Chlide *a* or Chl *a* to form Chlide *f* or Chl *f* is a four-electron oxidation (Ho et al. 2016). Gary et al. (2017) have suggested that the oxygen atom of the 2-formyl group of Chl *f* is derived from dioxygen. However, this conclusion may disagree with the observation that light is required and the possibility that ChlF might predate the origin of PsbA and thereby PSII (Ho et al. 2016). In principle, four electrons might be extracted from the substrate molecule after absorption of four photons, and considering that ChlF is sensitive to DCMU, those electrons are probably transferred to plastoquinone. If all four electrons were extracted by photooxidation, then water could potentially provide the oxygen for formation of the formyl group. These observations might be

reconciled if both light and oxygen are somehow required for this reaction, but for now, the resolution of these issues will likely require the development of an *in vitro* assay for Chl *f* synthase activity. However, to date, we have been unable to identify appropriate conditions for the study of this reaction *in vitro*.

With the exception of a PsbA paralog in *Gloeobacter* sp. with unknown function, ChlF sequences form the earliest diverging clade of the PsbA family, which presumably arose by a series of gene duplications followed by sequence divergence (Murray 2012; Cardona et al. 2015). The ChlF sequences differ substantially from those of PsbA throughout the entirety of the polypeptide, but specifically ChlF has an N-terminal extension, does not appear to be processed at the C-terminus, and lacks all but one of the key C-terminal residues required to ligate the $Mn_4Ca_1O_5$ cluster that is essential for water oxidation by PSII (Murray 2012; Cardona et al. 2015; Ho et al. 2016). Residues for binding Chl *a*, including P680, however, are conserved, as are the histidine ligands to the non-heme Fe atom (Murray 2012; Ho et al. 2016). As shown here and as predicted from its sequence, purified ChlF binds Chl *a*, Pheo *a*, and carotenoids. The observed properties of ChlF presumably reflect functional differences from those of PsbA (D1) in PSII (Murray 2012; Cardona et al. 2015; Ho et al. 2016). Moreover, it remains a possibility that ChlF is the progenitor of the PsbA subunits of PSII that can bind $Mn_4Ca_1O_5$ clusters (Ho et al. 2016). Except for the unusual protein of unknown function in *Gloeobacter* sp. noted above, only the gene duplication that gave rise to PsbD led to sequences that are more divergent from PsbA than ChlF (Murray 2012; Cardona et al. 2015). Interestingly, unlike the heterodimeric PsbA/PsbD cores of PSII and the PufL/PufM cores of bacterial reaction centers, ChlF forms homodimers, which may represent an ancestral state compared to more complex type-2 reaction centers. From an evolutionary perspective, the formation of heterodimeric proteins from homodimeric ancestors must be driven

by evolutionary forces related to specific differences in biological functions, which can select for and optimize the propensities to dimerize as well as the dynamics of protein-protein interface interactions (Jones and Thornton, 1996).

To determine whether ChlF might interact with PsbD (D2) or other components of PSII, a PsbD-less strain of *Synechococcus* 7002 was constructed by deleting the *psbD1* and *psbD2* genes. Consistent with previous reports concerning the mutagenesis of the *psbD* genes in *Synechococystis* sp. PCC 6803 (Yu and Vermaas 1990), deletion of the *psbD1* and *psbD2* genes in *Synechococcus* 7002 resulted in the loss of PSII activity. However, when the $chlF^{7521}$ gene was expressed in this strain lacking PsbD and PSII activity, Chl *f* production still occurred (**Fig. 5**). Notably, even higher levels of Chl *f* accumulated in this strain compared to those when this gene was expressed in cells of WT *Synechococcus* 7002 (**Figs. 1 and 5**). The obvious conclusions from these findings are that Chl *f* synthesis is neither dependent upon the presence of PsbD (D2) nor upon the presence of PSII activity or intact PSII in cells. One possible reason for the apparently enhanced ChlF activity could be that more substrate, either Chl *a* or Chlide *a*, is available for modification by Chl *f* synthase when PSII cannot be correctly assembled. This hypothesis led us to test whether Chl *f* synthesis might also be enhanced in an *ycf4* mutant that is impaired in PSI assembly (Boudreau et al. 1997; G. Shen and D. Bryant, unpublished results). However, when $chlF^{7521}$ was expressed in an *ycf4* mutant of *Synechococcus* 7002, the Chl *f* levels were similar to those produced in WT cells (data not shown). This result shows that defective PSI assembly neither positively nor negatively affects ChlF activity in *Synechococcus* 7002.

As noted above, the His ligands for Chl *a* molecules P680 and ChlZ are conserved in ChlF, as are the His ligands to the non-heme iron atom and most residues for the Q_B-binding site, all of which are key functional elements of PsbA (see the multiple sequence alignment of ChlF (PsbA4)

proteins and PsbA (D1) proteins in Fig. S2 of Ho et al. 2016). Flash photolysis of ChlF produced a relatively long-lived Chl *a* triplet that could be observed by both optical spectroscopy and EPR (**Figs. 6 and 7**). The sensitivity of Chl *f* synthesis to the PSII inhibitor DCMU strongly suggests that Chl *f* binds plastoquinone as an electron acceptor *in vivo* (**Fig. 5**). However, when the equivalent of the Tyr_Z in PSII (Tyr residue 183) was mutated to Phe, *Synechococcus* 7002 cells expressing the Y183F variant protein actually accumulated about 1.9-fold more Chl *f* compared to cells expressing WT $ChlF^{9212}$. This result indicates that ChlF is likely to have substantially different electron transfer reactions and mechanism in comparison to the reactions associated with PsbA in PSII.

Because of the mechanistic details for activation of transcription of the FaRLiP gene cluster during acclimation to FRL, cyanobacteria that perform FaRLiP can only synthesize Chl *f* when cells are grown in FRL (Gan et al. 2014, 2015; Zhao et al. 2015; Ho et al. 2016, 2017a). However, when the *chlF* gene is heterologous expressed in *Synechococcus* 7002, the *chlF* gene is not subject to regulation by RfpABC and thus Chl *f* synthase activity can occur in white light. Thus, it was of some interest to determine whether ChlF activity might be influenced by light quality in the heterologous system. When cells lacking PsbD and PSII and expressing $chlF^{7521}$ were grown under LL, RL, GL, or FRL conditions, Chl *f* levels were lowest in LL, intermediate in RL or GL, and highest in cells grown in FRL. The *Synechococcus* 7002 cells grown in FRL accumulated less Chl *a* than cells grown under the other light conditions, but individual cultures in which Chl *f* represented 3 to 4 % of the total Chl were usually obtained. These Chl *f* levels are only about half those achieved by cyanobacterial cells undergoing FaRLiP. Additional studies will explore whether Chl *f* levels of this magnitude can positively affect growth of *Synechococcus* 7002 in FRL.

If introduced into crop plants, the capacity to synthesize FRL-absorbing Chls such as Chl *d* and/or Chl *f* might expand the light wavelength range that could be used to support oxygenic photosynthesis (Chen and Blankenship 2011; Chen 2014; Gan et al. 2014). Considering this idea, it was of interest to determine whether the Chl *f* that was synthesized in *Synechococcus* 7002 was actually associated with PSI complexes in the cells. PSI, which naturally binds 96 Chl *a* molecules per monomer in *Thermosynechococcus vulcanus* (Jordan et al. 2001) and presumably similar numbers in other cyanobacteria, contained Chl *f* at about the same proportion as measured for the total Chl content of the cells. This suggests that most of the Chl *f* that was produced was actually associated with PSI complexes in the PsbD-less and PSII-less cells expressing the *chlF*^{7521} gene. These Chl *f* molecules extended the FRL absorption of the PSI complexes slightly into the far-red region and caused the low-temperature fluorescence emission of the complexes to be slightly red-shifted compared to WT PSI complexes containing only Chl *a* (**Fig. 8**). However, compared to PSI complexes isolated from *F. thermalis* 7521 cells grown in FRL, which contain about 8% Chl *f*, neither the absorption nor the fluorescence emission of the *Synechococcus* 7002 PSI complexes was red-shifted to such a great extent. Future studies will be directed towards ascertaining whether the Chl *f* molecules inserted into heterologous PSI complexes are functional in energy transfer and whether they affect the trapping efficiency of PSI complexes for PSI activity and cell growth in *Synechococcus* 7002.

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Figures

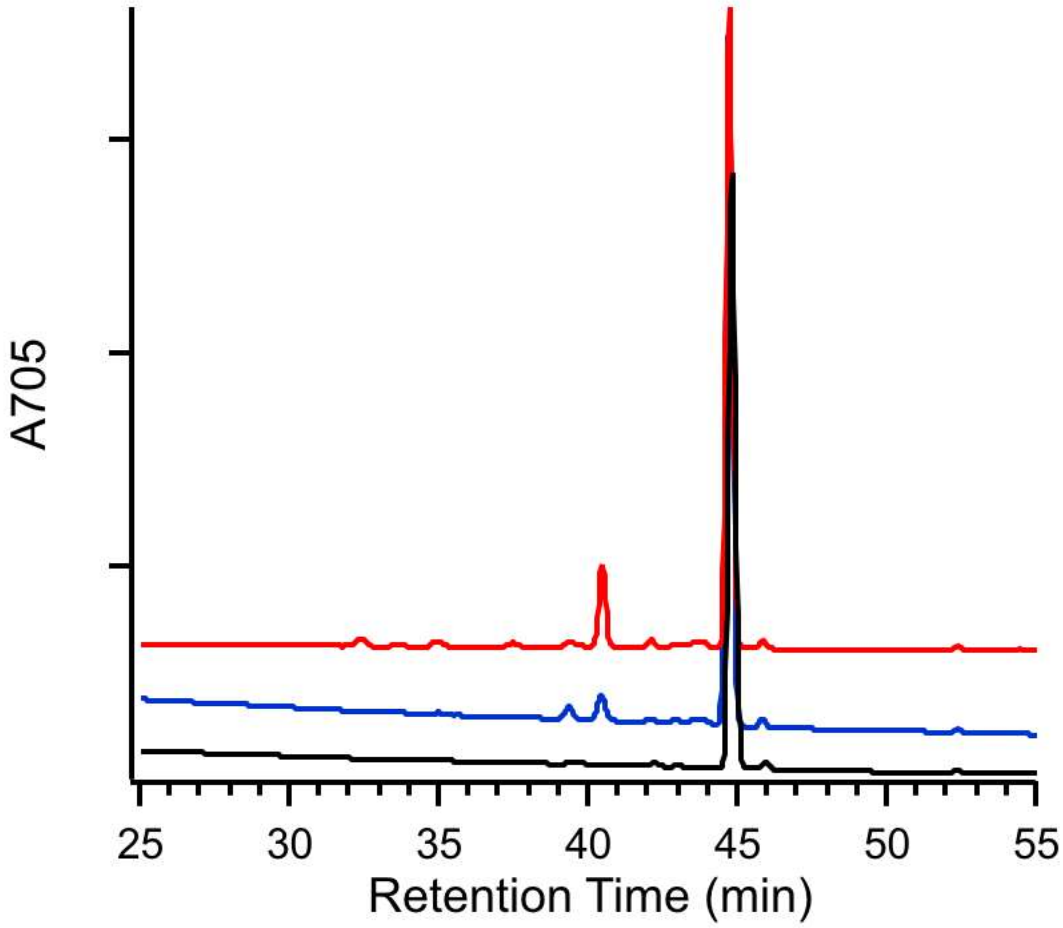

Fig. 1 Heterologous expression of *chlF*^{7521} leads to higher amounts of Chl *f* in *Synechococcus* 7002 than from *chlF*^{9212}. Based on OD_{750}, equal numbers of cells, which had very similar Chl *a* contents, were used for the analysis. The elution profile at 705 nm for reversed-phase HPLC analysis of pigments extracted from cells of *Synechococcus* sp. PCC 7002 wild-type strain (black line) and strains Ex.*::chlF*^{9212} (blue line) and Ex.*::chlF*^{7521} (red line) that express the *chlF* genes from FaRLiP strains *C. fritschii* 9212 *and F. thermalis* 7521, respectively. The elution positions of Chl *f* (40.5 min) and Chl *a* (45 min) are indicated. For additional details, see text.

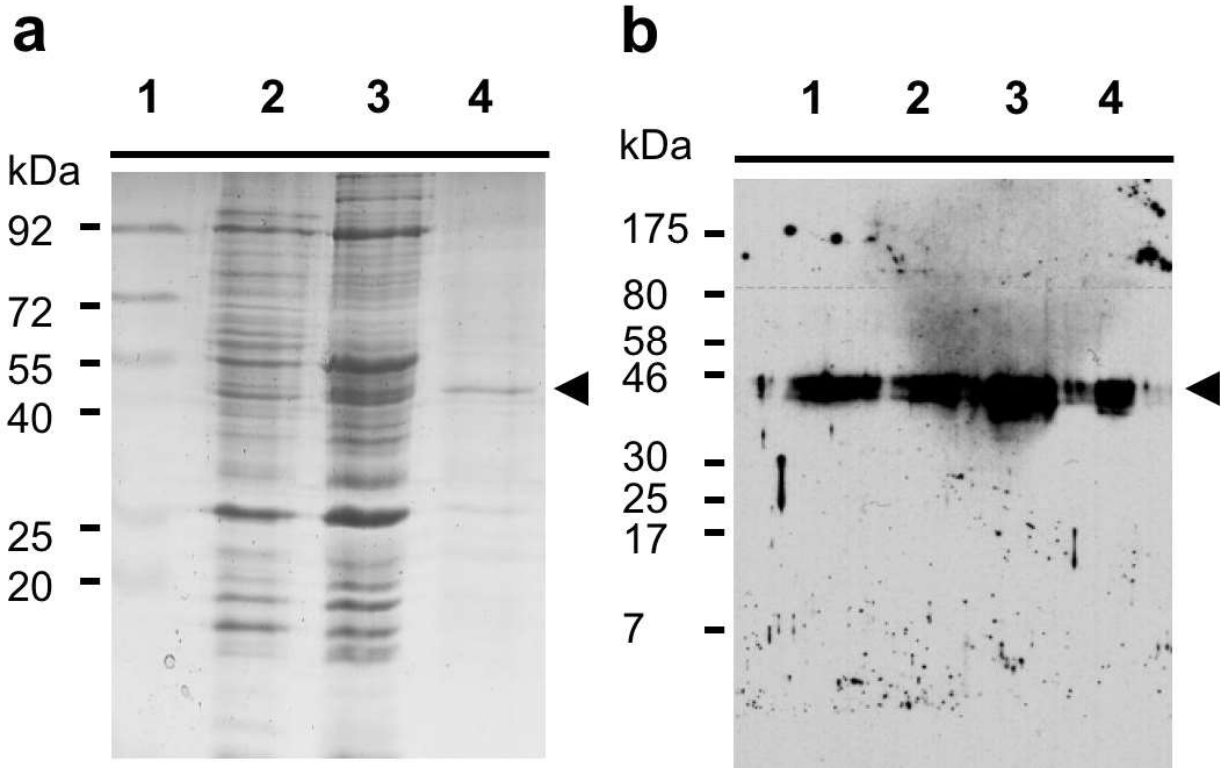

Fig. 2 Purification and verification of ChlF^{7521}. **a** SDS-PAGE analysis of *Synechococcus* 7002 membranes and purified ChlF^{7521}; the gel was stained with Coomassie blue. Lane 1, protein molecular mass markers; lane 2, thylakoid membranes isolated from wild-type *Synechococcus* 7002; 3, thylakoid membranes of strain Ex.*::chlF*^{7521}; and lane 4, purified [His]$_{10}$-tagged ChlF^{7521}. **b** Immunoblotting detection of [His]$_{10}$-tagged ChlF^{7521} from four independent preparations produced by IMAC. Lane 1, ChlF_prep #1; lane 2; ChlF_prep #2; lane 3 ChlF_prep #3; and lane 4, ChlF_prep #4. A polypeptide of ~44 kDa was detected in all four preparations.

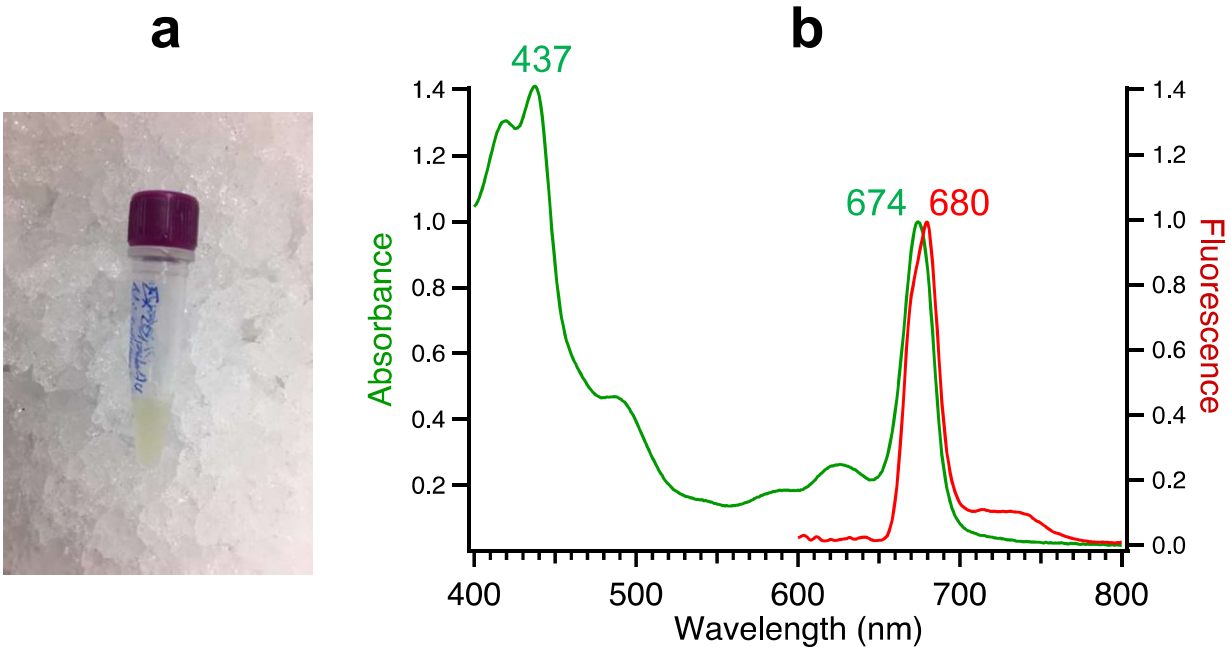

Fig. 3 ChlF^{7521} is a Chl-binding protein. **a** Appearance of as-purified ChlF^{7521}; the protein solution is yellowish-green in color. **b** Absorption spectrum (dark green line) and low-temperature (77 K) fluorescence emission spectrum (red line) of purified ChlF. The excitation wavelength was 440 nm. The spectra were normalized at 674 nm/680 nm as shown, and thus the absorbance and fluorescence values are relative units.

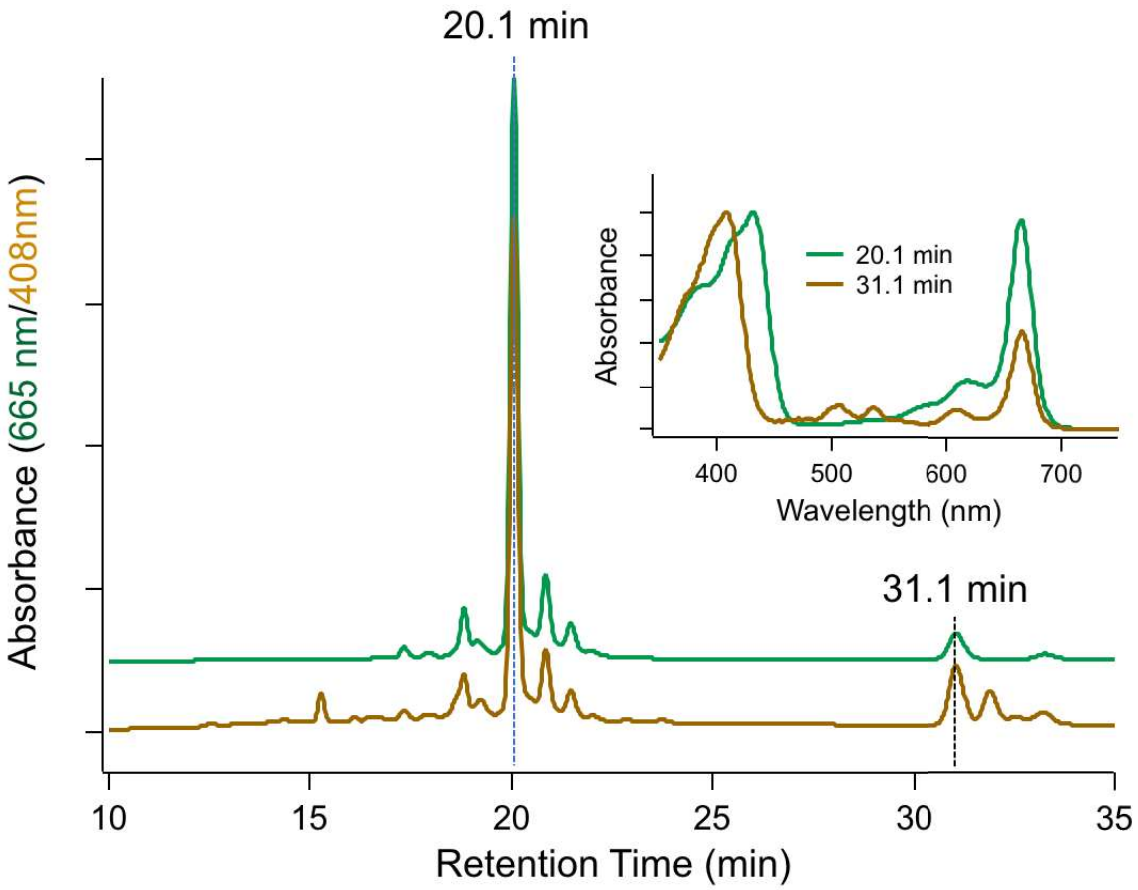

Fig. 4 Pigment content of purified ChlF^{7521}. Reversed-phase HPLC elution profiles of a pigment extract from purified ChlF^{7521} monitored at 665 nm (green line) and 408 nm (brown line). The insert shows the in-line absorption spectra of Chl *a* eluting at 20.1 min (green line) and Pheo *a* eluting at 31.1 min (brown line). The minor peaks at about 15.5 and 32 min are zeaxanthin and β-carotene, respectively (also see Fig. S2), and the minor peak at about 18.5 minutes is Chl *f*. For additional details, see text.

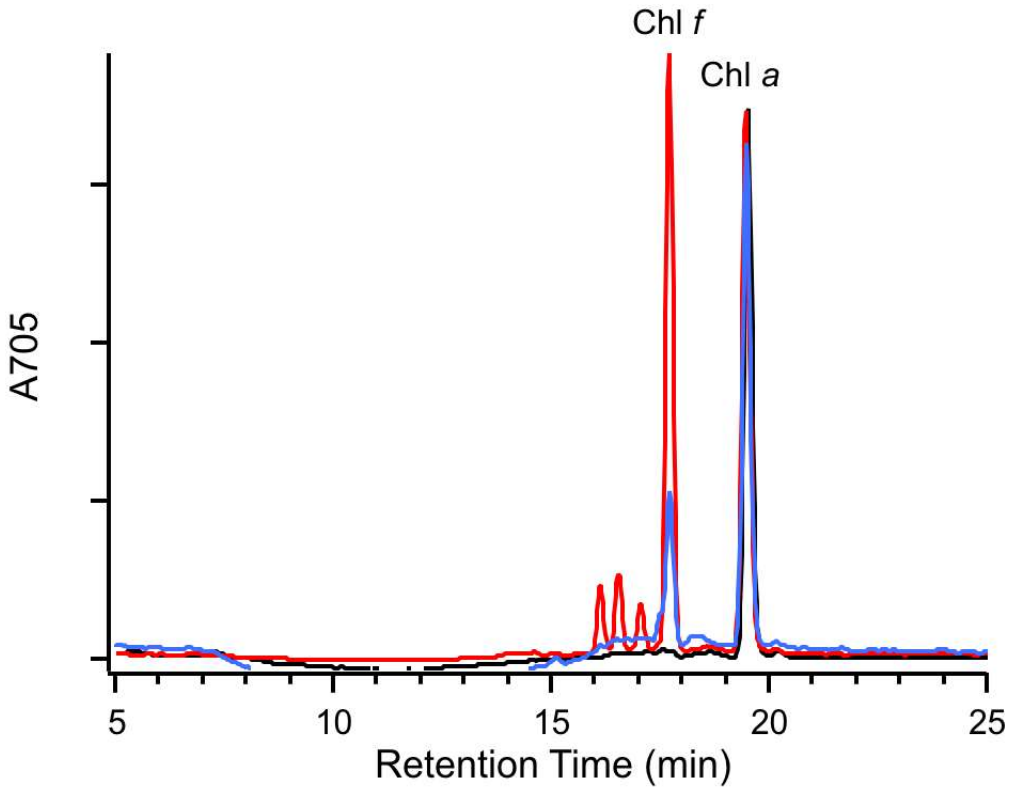

Fig. 5 DCMU inhibits the production of Chl *f* in *Synechococcus* 7002. Reversed-phase HPLC elution profiles at 705 nm are shown in the figure to assess Chl *f* production in the *Synechococcus* 7002 *ΔpsbD1 ΔpsbD2* mutant strain (PsbD-less) expressing *chlF*^{7521}. Based on OD_{750}, pigments were extracted from equal numbers of cells of the PsbD-less (*ΔpsbD1 ΔpsbD2*) double mutant strain of *Synechococcus* 7002 (control; black line); the *Synechococcus* 7002 *ΔpsbD1 ΔpsbD2* Ex*::chlF*^{7521} strain (red line); and the *Synechococcus* 7002 *ΔpsbD1 ΔpsbD2* Ex::*chlF*^{7521} strain treated with 10 μM DCMU (blue line). DCMU addition inhibited the production of Chl *f* but had little or no effect on the synthesis of Chl *a* because the cells were growing photoheterotrophically under these conditions. Note that the PsbD-less mutant produces much more Chl *f* than is produced by expression of *chlF*^{7521} gene in wild-type cells of *Synechococcus* 7002 (compare the relative Chl *f* and Chl *a* peak areas with those in **Fig. 1**).

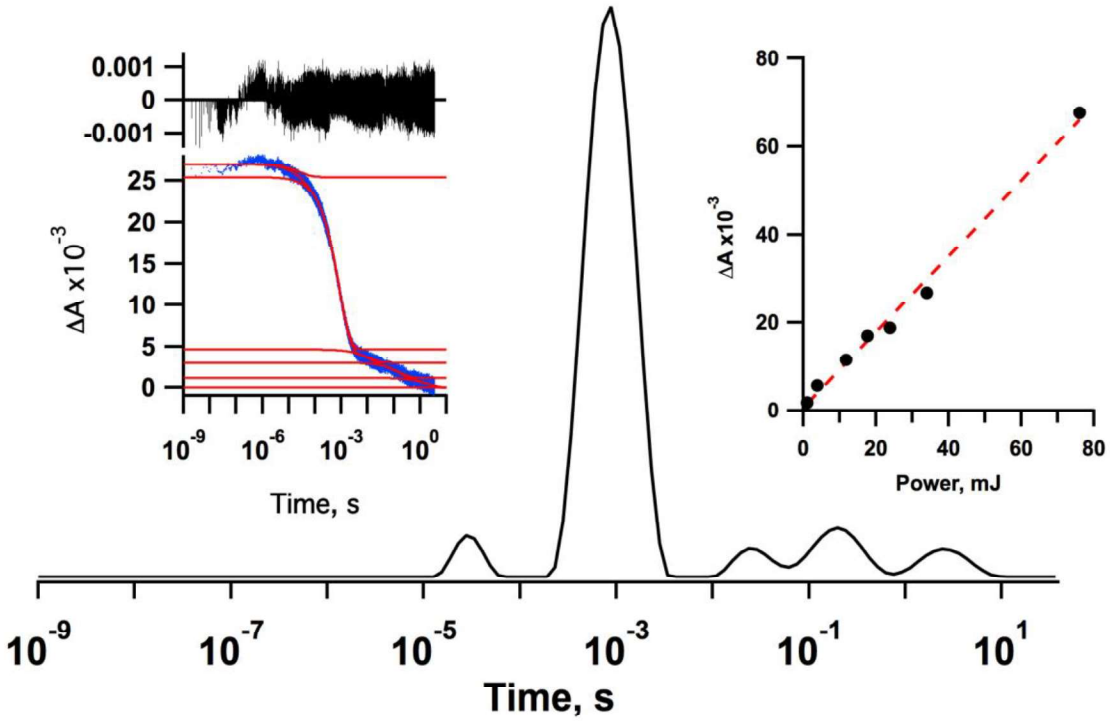

Fig. 6 Transient absorbance kinetics measured at 830 nm of purified ChlF[7521] following a laser flash. The main plot is the CONTIN decomposition of the experimental data (shown in blue points in the inset on the left) showing a major exponential decay component (817 µs) and four minor exponential decay components (28 µs, 11 ms, 161 ms, and 2.1 s). The solid red lines in the inset, left represent the resolved kinetic components from the CONTIN decomposition superimposed on the experimental data. The upper panel shows the residuals, i.e. the difference between the experimental points and the fitted curves. The inset on the right shows the signal amplitude at the laser powers depicted.

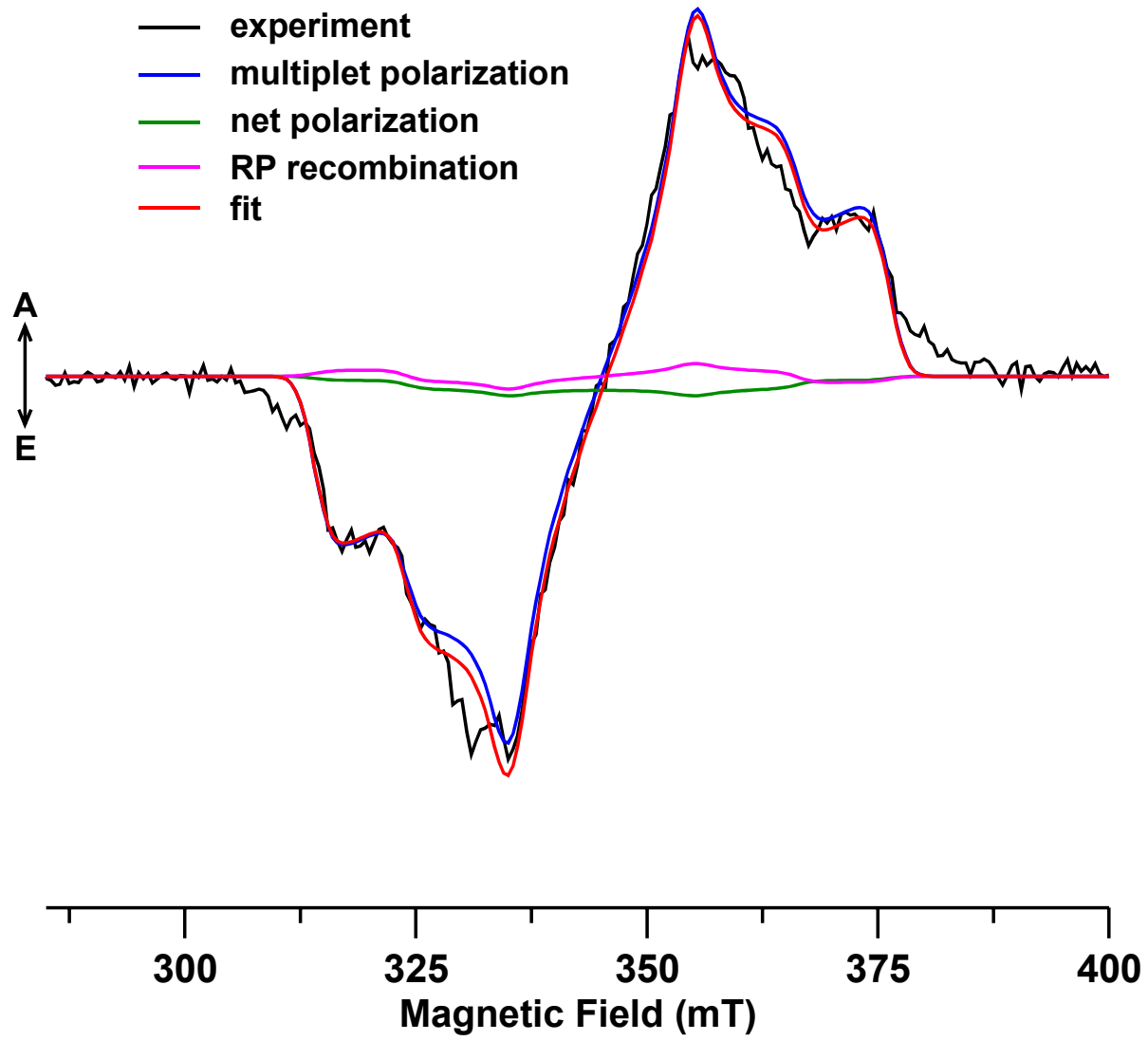

Fig. 7. Transient EPR spectrum of ChlF^{7521} at 90 K in the presence of 10 mM sodium ascorbate. The black trace is the experimental data (128 averages; microwave power, 17 μW); the red trace is a fit of the experimental spectrum calculated with D = 2.71 × 10^{-2} cm^{-1} and E = 3.3 × 10^{-3} cm^{-1}. The fitted spectrum is the sum of the blue, green and pink spectra. The blue trace is the multiplet polarization generated by ISC calculated as described in (Kandrashkin et al 2006) with $\kappa_{\|}$ = 1.1 and $\kappa_{\perp}$ = 0.40, which corresponds to p_x:p_y:p_z =0.18:0:0.82. The green trace is the net polarization generated by ISC and the pink trace is the polarization generated by radical pair recombination.

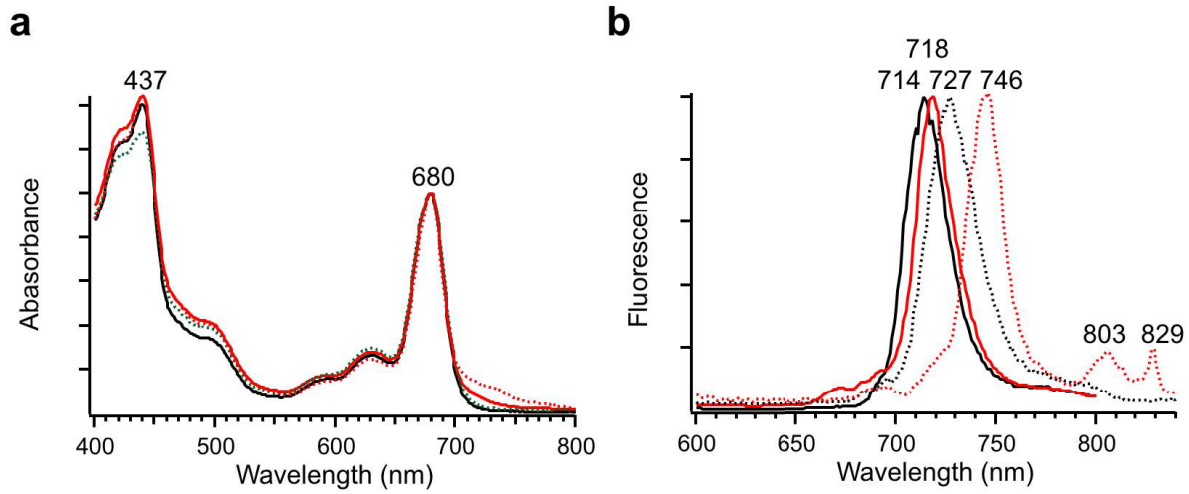

Fig. 8. Heterologously produced Chl *f* binds to *Synechococcus* 7002 PSI complexes**.** Absorption spectra (**a**) and fluorescence emission spectra at 77 K (**b**) of isolated PSI complexes from wild-type *Synecococcus* 7002 (solid black line), strain Ex*::chlF*^{7521} (solid red line), *F. thermalis* 7521 cells grown in WL (dotted black line) and *F. thermalis* 7521 cells grown in FRL (dotted red line). The excitation wavelength was 440 nm. Note the increased absorption between 700 and 750 nm for PSI complexes from strain Ex*::chlF*^{7521}and their red-shifted emission, which is not as extreme as for the Chl *f* in the FRL-PSI complexes from *F. thermalis* 7521.

Table 1. List of plasmid constructs and cyanobacterial strains used in study.

Plasmid constructs	Characteristics	Reference
pAQ1Ex-P_{cpcBA}	pAQ1-based expression vector; Gm^R	Xu et al. 2011
pAQ1Ex*::chlF*^{9212}	*chlF*^{9212} gene inserted in pAQ1Ex-P_{cpcBA}; Gm^R	Ho et al. 2016
pAQ1Ex*::chlF*^{7521}	*chlF*^{7521}gene inserted in pAQ1Ex-P_{cpcBA} ; Gm^R	This study
pAQ1Ex*::chlF*^{9212}(Y183F)	Tyr183 to Phe mutant of *chlF*^{9212} gene inserted in pAQ1Ex-P_{cpcBA} ; Gm^R	This study
ΔpsbD1::aadA	pUC19 with *psbD1* region of *Synechococcus* 7002 genome and *psbD1* replaced by *aadA;* Sp^R	This study Gingrich et al. 1990
ΔpsbD2::aphAII	pUC19 with *psbD2* region of *Synechococcus* 7002 genome and *psbD2* replaced by *aphAII;* Km^R	This study Gingrich et al. 1990
Cyanobacterial Strains		
Synechococcus sp. PCC 7002	Non-FaRLiP strain; model cyanobacterium	Rippka et al. 1979
Fischerella thermalis PCC 7521	FaRLiP strain	Rippka et al. 1979
Chlorogloeopsis fritschii PCC 9212	FaRLiP strain	Gan et al. 2015
Strains of *Synechococcus* sp. PCC 7002		
Ex*::chlF*^{9212}	*Synechococcus* 7002, pAQ1Ex*::chlF*^{9212}; Gm^R	Ho et al. 2016
Ex*::chlF*^{7521}	*Synechococcus* 7002, pAQ1Ex*::chlF*^{7521}; Gm^R	This study
Ex*::chlF*^{9212} (Y183F)	*Synechococcus* 7002, pAQ1Ex*::chlF*^{9212} (Y183F); Gm^R	This study
Synechococcus 7002 *ΔpsbD1 ΔpsbD2* (PsbD-less)	*ΔpsbD1*::*aadA ΔpsbD2*::*aphAII;* Km^R, Sp^R Mutant lacking PsbD; PSII-less	This study
Synechococcus 7002 *ΔpsbD1 ΔpsbD2,* Ex*::chlF*^{7521}	Overexpression of *chlF*^{7521} in mutant lacking PsbD (*ΔpsbD1 ΔpsbD2*); Km^R, Sp^R, Gm^R	This study